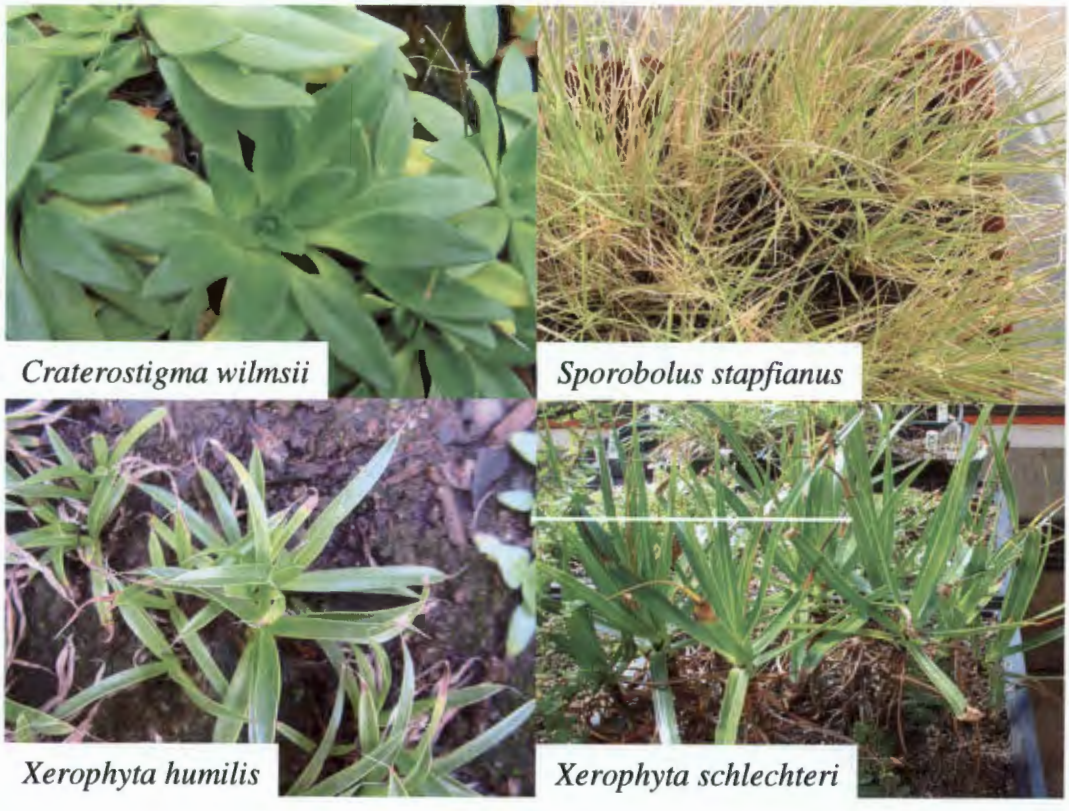
Stretched to the limit: Leaf tensile properties and lignin content of resurrection plants



Craterostigma wilmsii | *Sporobolus stapfianus*

Xerophyta humilis | *Xerophyta schlechteri*

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Abstract

Leaf tensile strength was measured for four resurrection plants, *Craterostigma wilmsii* Engl, *Xerophyta schlecteri* (Baker) N.L. Menezes, *Xerophyta humilis* (Baker) T. Durand & Schinz and *Sporobolus stapfianus* Gandoger, as well as two desiccation-sensitive controls, *Zea mays* L. and *Arabidopsis thaliana* (L.) Heynh. (ecotype Columbia) at full hydration and after dehydration, both on the plant (naturally-dried) and rapidly off the plant causing death (flash-dried). In the desiccation-tolerant plants, leaf tensile strength was higher in the monocots than the dicots at full hydration. Three different mechanisms of cell protection occur in resurrection plants on drying: cell-wall folding, packing vacuoles with non-aqueous solute or a combination of the two. Tensile strength in *C. wilmsii* (dicot) increased when naturally-dried but decreased when flash-dried, possibly due to the nature of the drying mechanisms (wall folding). The, leaf tensile strength of the *Xerophyte* species, both monocots, increased when naturally dried and when flash-dried. *Xerophyte* species pack their vacuoles during desiccation. *S. stapfianus*, a grass which uses a combination of wall folding and vacuole packing, had the highest tensile strength possibly due to its unique architectural structure. Differences in leaf architecture, in terms of lignin content, were examined using light microscopy after histo-chemical staining for lignin, which showed that monocotyledons had a higher percentage of lignin per unit leaf cross-sectional area than dicotyledons. A regression analysis revealed that leaf tensile strength and lignin content were positively correlated in fully hydrated leaves, but no relationship existed between lignin content and naturally dried leaves. This may be due to variations of protective mechanisms induced during desiccation by the four resurrection plants. Notching was observed in *X. schlechteri*, behaving differently to grasses which are notch-insensitive, possibly due to large lignin contents on the outer edges of the leaves.

Introduction

It has been suggested that the tensile properties of leaves may be useful in studying the response of plants to water stress, in particularly drought stress (Balsamo *et al* 2003a). In a study involving three grasses of the genus *Eragrostis*, each with a different drought tolerance, Balsamo *et al.*(2003a) showed that leaf tensile strength increased with drought tolerance. In a second study, Balsamo *et al.*(2003b) showed that in drought-tolerant grasses tensile strength increased as relative water content (RWC) decreased whereas the tensile strength of a desiccation-tolerant grass did not change as RWC decreased. Most studies on leaf tensile properties involve grasses (Balsamo *et al.*2003a; Balsamo *et al.* 2003b and Vincent 1983) but very little is known about the change in tensile properties of dicotyledonous plants and other monocotyledonous plants (both desiccation-tolerant and desiccation-sensitive).

Biomechanical properties of leaves – tensile strength

McGowan (1999) defines tensile strength as the maximum tensile force that a material can withstand prior to breaking per cross-sectional area at the break. The break as a result of a tensile force, referred to as a fracture, depends to some extent on the strength of the material. An Instron is typically used to measure the mechanical properties of materials, including tensile strength, however a tensilmeter can be built on a smaller scale providing a measure of tensile strength only (Martens & Booysen 1968). These authors have shown that a tensilmeter allows the tensile strength of grass leaves to be measured accurately.

How plant tissues are strengthened

The vascular bundle

Even though many morpho-anatomical aspects can influence the biomechanical properties of leaves, the vascular bundle and epidermis are most responsible for preventing damage caused by tensile stresses.

The vascular bundle is comprised of a variety of structurally and functionally different cell types. The most abundant cells are those of the xylem and phloem, and depending on

the species, there will be varying fractions of parenchyma and fibres. The xylem tissue contains tracheids, vessel members or both, which are dead when mature; xylem cells commonly have cell walls impregnated with lignin. The primary function of the xylem in the leaf is to transport water, solutes, even hormones, to the leaf lamina. The phloem tissue consists mostly of sieve tube members which function to transport the carbohydrates produced in the leaf to other parts of the plant. Fibres, associated with both xylem and phloem, belong to a class of plant tissue called sclerenchyma, which is dead at maturity, and have secondary cell walls thickened with cellulose and usually impregnated with lignin. The secondary function is mechanical stabilization based on the lignified xylem and the sclerified fibres.

Leaf venation and leaf strength

Large and mechanically stiff midribs are favourable in leaves as the greatest mechanical stress that a leaf encounters occurs along its longitudinal axis. In most dicotyledonous plants, the leaf lamina has lateral veins connected to a central midrib (Cutler 1978). The lateral veins form a network of minor and major veins referred to as reticulate venation (Cutter 1971). In monocotyledonous plants, such as grasses, the veins in the leaves are parallel. This parallel venation leads to a mechanically stable leaf.

Sclerophylly and leaf strength

Sclerophylly is due to an increase in sclerenchyma tissue in the vascular system. The leaves of sclerophyllous plants tend to be thick, tough and leathery increasing toughness, hardness and stiffness of the leaf.

Balsamo *et al.* (2003c) compared the leaf tensile properties between the dicotyledonous mesic deciduous tree *Prunus serrulata* and the dicotyledonous, xeric and sclerophyllous chaparral evergreen shrub *Heteromeles arbutifolia*, finding that tensile strength, toughness, modulus of elasticity and failure strain were all higher in the sclerophyllous evergreen.

Sclerenchyma is known to be the main, load-bearing fibrous tissue in grass leaves. Studies by Vincent (1982; 1991) showed that the stiffness and strength of leaves of *Lolium perenne* are linearly related to the amount of sclerenchyma in them, where 90-95% of the longitudinal stiffness of the leaf is due to this sclerenchyma.

Lignin and leaf strength

A study by Theron and Booysen (1968) on the factors influencing the breaking tension of various grass leaves proposed that the most important factor in determining tensile strength in immature grass leaves was the degree of lignification. Lignin is a major constituent in the walls of cells that provide mechanical support (sclerenchyma and vascular fibres) and transport water (tracheids and vessel members) (Niklas 1992). Lignin content and type varies with species, tissue, developmental stage and sub cellular localization (Lewis & Yamamoto 1990). Very little has been done on investigating the contribution of lignin to leaf tensile strength but it is generally thought that lignin strengthens tissues.

The relevance of biomechanics to desiccation tolerance

What is desiccation tolerance and drought tolerance?

There is much confusion about the difference between drought tolerance and desiccation tolerance. Alpert & Oliver (2002) define drought as any level of water availability that is low enough to reduce plant performance. Tolerance of drought indicates that a plant can tolerate low water availability but not low enough to cause desiccation (drying to equilibrium with the air).

Desiccation tolerance, on the other hand, is the ability to revive from the air-dried state (Oliver 1996). Many vascular plants are able to produce structures (seeds and pollen) that are desiccation tolerant, but very few have the ability to survive desiccation of their vegetative tissues to below 20% relative water content (Oliver 1996). These vascular plants that have the ability to 'resurrect' their vegetative tissues following rehydration are referred to as 'resurrection plants' (Proctor & Pence 2002). The most studied resurrection

plants are *Craterostigma plantagineum* (Bartels *et al.* 2001), *Craterostigma wilmisii* (Farrant 2000), *Sporobolos stapfianus* (Vecchia *et al.* 1998), *Myrothamnus flabellifolius* (Sherwin & Farrant 1996; Farrant & Kruger 2000) and various members of the genus *Xerophyta* (Farrant 2000 for *Xerophyta humilis*; Mundree & Farrant 2002 for *Xerophyta viscosa*).

Mechanisms of survival in desiccation tolerant plants

Studies have shown that resurrection plants rely on inducible cellular protective mechanisms against desiccation (Farrant *et al.* 1999). Farrant (2000) showed that *C. wilmsii, M. flabellifolius* and *X. humilis* protect, to varying degrees, against damage to the plasma membrane on dehydration by fragmenting their water filled vacuoles into several smaller non-aqueous vacuoles. The extent of vacuolation differs between these three resurrection plants (Farrant 2000). Farrant (2000) observed only a small amount in *C. wilmsii.*

Farrant and Sherwin (1998) have shown that some desiccation tolerant plants (such as *C. wilmsii*) fold their cell walls during drying. On rehydration, the cell resumes its normal shape. This strategy may help to reduce plasma membrane damage (Farrant & Sherwin 1998). Vicre *et al.*(1999) show that this wall folding is not a result of wall collapse, but is a carefully controlled process.

The accumulation of sugars occurs as one among the many metabolic changes occurring prior to desiccation, which limits damage to the sub-cellular environment (Alpert & Oliver 2002). As water is lost from the cells, the cytoplasmic components and cell contents become highly viscous. This may cause molecular interactions leading to protein denaturation and membrane fusion (Hoekstra *et al.* 2001). Sucrose has been shown to prevent these molecular interactions (Hoekstra *et al.* 2001). Two hypothesis exist explaining the contribution of sucrose to the survival of resurrection plants (discussed by Swayze 2004)

The first hypothesis, "the water replacement" hypothesis, suggests the replacement of water by sucrose in biological membranes. Water is important in maintaining the assembly of phospholipids in cell membranes and for the correct conformation of proteins (Vicre *et al.* 2003). On initial drying, sugars are excluded from membranes in favour of water, which forms a water shell maintaining their conformation and hydration (Hoekstra *et al.* 2001). As more water is lost from the cells, the water shell disappears and the sugar molecules act as a water substitute by replacing the strong hydrogen bonds usually present between water and the polar heads of the lipid bilayer, maintaining the stabilizing effect of water (Hoekstra *et al.* 2001).

The second hypothesis suggests that a combination of low water content and high sucrose levels causes the cytoplasm to become highly viscous causing a glassy state to form (Buitink *et al.* 1999 in Vicre *et al.* 2003). In the glassy state, the desiccated cell is brittle yet solid adding to the structural support of the cell and reducing mechanical damage (Hoekstra *et al.* 2002). The structural support provided by this glassy state could be valuable to the tensile strength of a desiccation-tolerant plant in the desiccated state.

The accumulation of sugars, and the subsequent stabilizing of the membrane or cytoplasm, the presence of non-aqueous vacuoles and the folding of cell walls may be important contributions to the tensile strength of the leaves of desiccation-tolerant plants. When rapidly dried, these mechanisms are absent, often leading to the death of the desiccation-sensitive plant (Farrant *et al.* 1999).

The effect of desiccation- and drought-tolerance on leaf biomechanical properties

An increase in drought tolerance correlates with an increase in tensile strength (Balsamo *et al.* 2003a). A study by Balsamo *et al.*(2003a) involving three grasses of the genus *Eragrostis* showed *E. curvula* to be the most drought-tolerant species in the study, *E. capensis* the most drought-sensitive species and *E. tef* to be somewhere in between. *Eragrostis curvula* had a higher tensile strength than *E. tef* which then has a higher tensile strength than *E. capensis*. Further studies by Balsamo *et al.*(2003b) on the tensile strength

of hydrated and air-dried plants of drought-tolerant *E. curvula* and the desiccation-tolerant *E. nindensis* showed that tensile strength increases with tissue dehydration in drought-tolerant *E.* curvula but leaf tensile strength remains the same in *E. nindensis* on desiccation.

Objectives

In this study the following hypotheses were tested: (1) the tensile strength of leaves of desiccation-tolerant plants do not change as (RWC) decreases; (2) the tensile strength in leaves is positively correlated with the percentage of lignin per unit area. The tensile strengths of leaves of six different plants species were determined - four resurrection plants and 2 desiccation sensitive plants (the desiccation-sensitive plants were used as a control) - when fully hydrated and at a RWC of less than 10%. Further, leaves were flash dried (Farrant *et al.* 1985) to allow a comparison of tensile strength under two different drying methods. A choice of monocotyledonous and dicotyledonous plants in this study should allow a suitable comparison of differences in tensile strength between the two venation patterns.

Studied species

Four desiccation-tolerant and two desiccation-sensitive plants were selected for this study. The selected desiccation-tolerant plants were *Craterostigma wilmsii* Engl, representing a dicotyledonous plant, and *Xerophyta schlecteri* (Baker) N.L. Menezes, *Xerophyta humilis* (Baker) T. Durand & Schinz and *Sporobolus stapfianus* Gandoger representing the monocotyledonous plants. The monocotyledonous species differ greatly in leaf size, where *S. stapfianus* < *X. humilis* < *X. schlechteri* in average leaf width and length.

Z. mays was selected as a drought-tolerant, yet desiccation-sensitive, monocotyledonous plant. Studies by Balsamo & Orkwiszewski (2004) have shown that leaf age in *Z. mays* influences tensile strength. As a *Z. mays* leaf develops, its tensile strength increases. By providing tensile strength values for varying ages of *Z. mays* leaf, this tested whether the constructed tensilmeter would give comparable and viable readings. Balsamo & Orkwiszewski (2004) went on to investigate lignification of *Z. mays* in which they found

the cuticle of juvenile *Z. mays* to contain lignin deposits and the vascular bundles, not the cuticle, of adult contained lignin.

Arabidopsis thaliana was used as a desiccation-sensitive dicotyledonous representative. Its leaf shape, size and growth form (i.e. rosette) are similar to *C. wilmsii*, providing a suitable comparison between the dicotyledonous plants. *A. thaliana* is also a well-studied plant in many fields.

Methods

Plant material

Craterostigma wilmsii Engl, *Xerophyta schlecteri* (Baker) N.L. Menezes, *Xerophyta humilis* (Baker) T. Durand & Schinz and *Sporobolus stapfianus* Gandoger were collected from the field and maintained in a glasshouse at the University of Cape Town (UCT) as previously described (Sherwin & Farrant 1996).

Zea mays L. and *Arabidopsis thaliana* (L.) Heynh.(ecotype Columbia) were grown from seed in potting soil. *Z. mays* was grown in a controlled growth room at UCT at 16 h light (140 μmol/m^2/s)/8 h dark cycle; 25°C; 40-60% relative humidity (RH). *A. thaliana* plants were grown at 16 h light (150 μmol/m^2/s)/ 8 h dark cycle; 22°C; 80-90% RH.

Mature leaves from all species were used for all experiments detailed below, with the exception of *Z. mays* for which immature leaves from 8-week-old plants were selected.

For all species, plants were watered to field capacity and covered with a transparent bag approximately 12 h prior to all measurements of fully hydrated plants. Naturally-dried plants (<10% RWC) were not watered for at least 21 days prior to taking measurements.

Fully hydrated leaves of all species were detached and placed in flash drying apparatus (Farrant *et al.* 1985). The leaves were placed on nylon mesh through which dry air (passed through silica gel) was blown from beneath the leaves for 24h. The RWC of leaves upon measurement was <10% RWC.

Tensile strength measurements – leaf morphology & mechanical properties

Leaf tensile strength measurements were recorded using a tensilmeter assembled using 1000, 2500 and10000 g Pesola scales (Barr, Switzerland), clamps, a metal stand, clamps and a container (Figure 1). The basal section of the *X. humilis, X. schelecteri, Z. mays* and

S. stapfianus leaf were always removed and cut into segments approximately 4 cm long. This is important as Balsamo *et al* (2003b) have shown that leaf tensile strength, elastic modulus and toughness decrease from leaf base to tip. The whole leaf of *C. wilmsii* and *A. thaliana* were used intact (leaf lengths were approximately 4cm in total). The leaf section of, or entire, leaf was secured between the clamps, with the basal end facing upwards, and South African five cent coins were added to the beaker one at a time until the leaf fractured between the clamps (see Figure 1). The value on the Pesola scale gave the sum of the failure load for the leaf and that of mass of the upper clamp (25 g). These values were converted to force (Newtons) by multiplying by gravity (9.8 m/s).

The thickness and width at the point of fracture for all leaves, with the exception of *S. stapfianus,* were measured using a Promax digital caliper (Fowler instruments, Boston, MA; USA). The cross-sectional areas of the naturally-dried and flash-dried *S. stapfianus* leaves were measured using AxioVision 2.05 software (Carl Zeiss Vision GmbH, Hallbergmoos, Germany) from digital images captured with an Axiocam digital camera (Zeiss, Hallbergmoos, Germany) of the fractured ends of this grass on a dissecting microscope (Wild Photomakroscop M400, Heerbrug, Germany). These leaves were too curled to measure with calipers.

Tensile strength was calculated by dividing the failure load by the cross-sectional area at the fracture (N/mm^2=MPa).

Between five and 10 replicate leaves were measured for each species and treatment from at least two different plants per species/treatment, except for *S. stapfianus* for which there were only two individual plants available (one hydrated, one dehydrated).

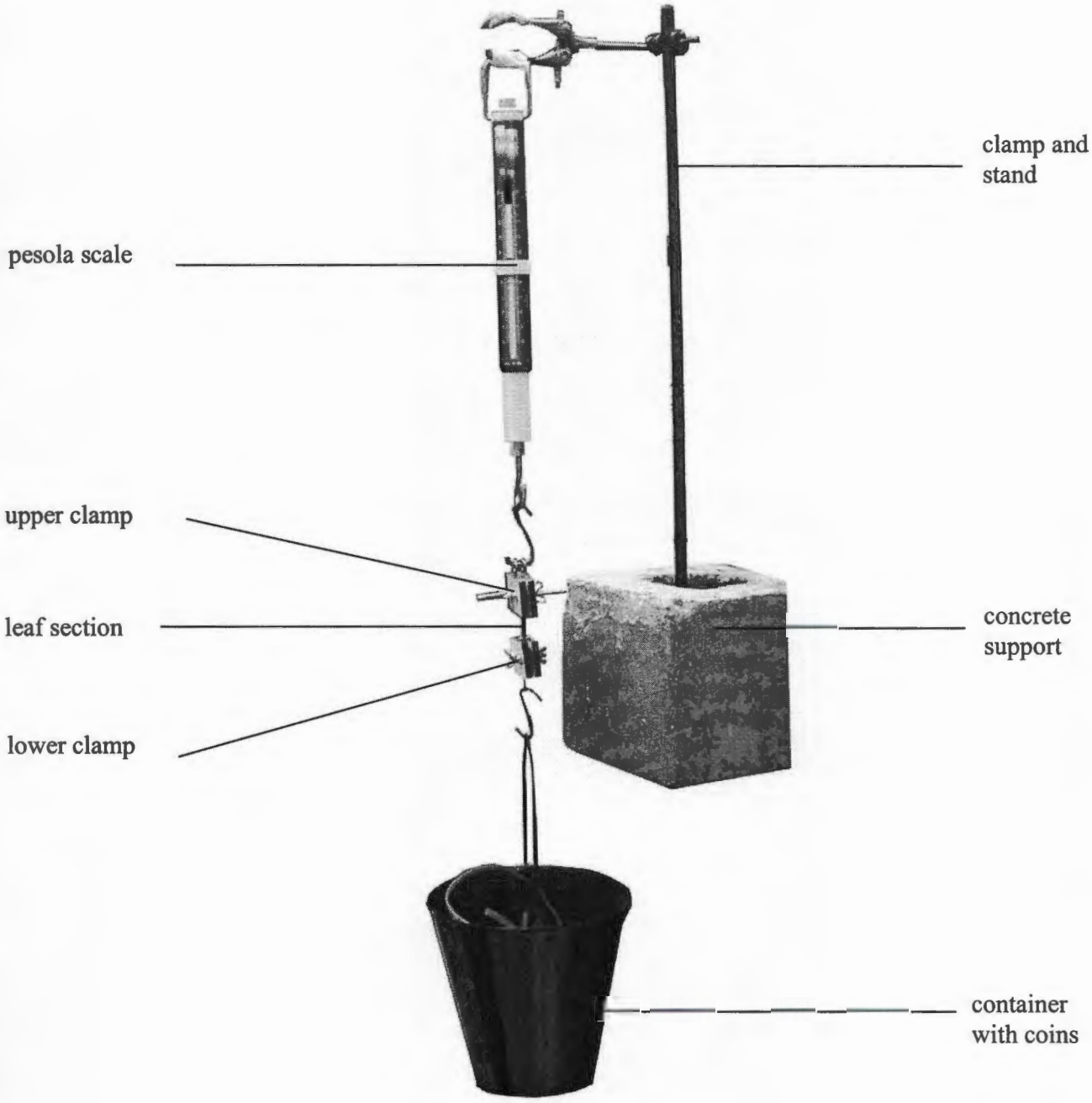


Figure 1: Side view of the portable tensilmeter

Relative water content

Measurements of relative water content (RWC) of leaf tissues were used to evaluate the water status of a plant. After fracture the fractured leaf segment in the upper clamp (basal end of leaf) was removed and immediately weighed on an AG135 Mettler Toledo balance (Columbus, Ohio, USA), which gave the fresh mass (FM) of the leaf section. After weighing, the leaves were placed in a Petri dish containing silica gel and placed in a 70°C oven for 47-51 h, in order to obtain the dry mass (DM). The moisture content (MC) of each leaf was calculated using the following equation:

$$MC\ (g\ H_2O/g\ DM) = (FM-DM)/DM$$

The RWC for each leaf was calculated using the following equation:

$$RWC(\%) = [(MC_{(fully\ hydrated\ leaf)} - MC_{(naturally/flash\ dried\ leaf)}) / MC_{(fully\ hydrated\ leaf)}] * 100$$

It was assumed that a fully hydrated leaf was at full turgor.

Sectioning and staining

Wax embedding

After fracture, the fractured leaf section in the lower clamp (proximal end of leaf) was fixed in 2.5% gluteraldehyde overnight. Following fixation, the leaf sections were dehydrated in the following ethanol gradient, for 4 h in each solution: 50% ethanol, 50% ethanol, 70% ethanol, 70% ethanol, 96% ethanol, 96% ethanol, absolute isopropanol, absolute isopropanol, absolute butanol, absolute butanol. Following dehydration the sections were impregnated with molten paraffin wax at 58 °C for 48 h, which was replaced with fresh wax after 24 h. The wax replaces any water in the tissues. The sections were removed from the wax and placed in stainless steel moulds filled with molten wax. The sections were orientated in the mould presenting the cross-sectional area of the leaf for the cutting surface. The moulds were cooled in ice and once the wax was solidified, removed from the mould.

Slide preparation and sectioning

Glass slides were washed in dilute sodium hydroxide and rinsed well in distilled, dionized water. The slides, once dried, were coated with Haupt's adhesive (Johansen 1940) and allowed to air-dry. Thin sections of embedded tissue (12 μm thick) were cut on a Leica Reichart Ultracut S rotary microtome (Vienna, Austria) and mounted on the coated slides.

Dewaxing,rehydrating and staining

The slides are placed in the following solutions for 10 minutes each (xylene to remove the wax and a decreasing ethanol gradient to rehydrate the tissues):
Absolute xylene, absolute xylene, absolute ethanol, absolute ethanol, 96% ethanol, 80% ethanol, 70% ethanol, 50% ethanol and distilled water for 20 min.

The slides were briefly stained with 1% aqueous Toluidine blue solution, staining lignified tissue blue and non-lignified tissue purple.

Light microscopy – lignin analysis

Stained sections were viewed with an inverted light microscope (Nikon, Tokyo, Japan) and images were captured with an Axiocam digital camera (Zeiss, Hallbergmoos, Germany) using AxioVision 2.05 software (Carl Zeiss Vision GmbH, Hallbergmoos, Germany). Axiovision software was used to measure the total cross-sectional area of the photographed leaf section and the cross-sectional area of lignin per leaf section. The percentage of lignin per unit area was then calculated for each species. There were up to five replicates for each species (both naturally-dried and hydrated).

Statistical analyses

Results were analysed using STATISTICA version 6.1 ANOVA, Tukey's HSD and Students t-tests where appropriate. A regression analysis between tensile strength and % lignin/unit area of wet plants was determined.

Results

Tensile strength

The mean tensile strength of the leaves of the desiccation-tolerant monocotyledonous species behaved in two different ways. The mean tensile strength of the leaves of *X. humilis* and *X schlecteri* increased from fully hydrated to both naturally dried and flash dried. Conversely, the mean tensile strength of *S. stapfianus* leaves remained the same when naturally dried but increased when flash dried. The mean tensile strength of the control, *Z. mays*, did not change when RWC decreased (both naturally dried and flash dried) (Table 1).

In the dicotyledonous species the mean tensile strength of leaves of *C. wilmsii* increased from fully hydrated to naturally dried but decreased from fully hydrated to flash dried. The mean tensile strength of the control, A. *thaliana*, decreased when RWC decreased (both naturally dried and flash dried) (Table 1).

Comparisons of the mean tensile strength between all species in the study when fully hydrated revealed that, in general, the mean tensile strength of the monocotyledonous species was higher than those of the dicotyledonous species. Additionally, the mean tensile strength of desiccation-tolerant leaves was higher than desiccation-sensitive control leaves of the same architectural structure. The trend in mean tensile strengths when fully hydrated and naturally dried was *S. stapfianus* > *X. humilis* > *X. schlecteri* > *Z. mays* > *C. wilmsii* > *A. thaliana* (Figure 2).

Lignin analyses

The leaves from all the species in the study exhibit some degree of lignification as indicated from Toulidine blue staining (Figure 3). Toulidine blue stains non-lignified tissue/cell walls purple and lignified tissue/cell walls blue. Dense areas of lignified tissue were seen in the tips of leaves of the desiccation-tolerant monocotyledonous plants from the *Xerophyta* species but not the desiccation-tolerant grass *S. stapfianus*. *S.stapfianus* had an unusually high degree of lignification in the epidermal cells.

There was a positive correlation between % lignin/ unit cross-sectional area leaf and tensile strength at full hydration (Figure 4). No linear relationship existed between leaf tensile strength and % lignin/ unit cross-sectional area for leaves when naturally dried.

Table 1: The within species variation of mean tensile strength values (± SE) (MPa) for leaves of all species at full hydration (RWC = 100%) and after dehydration (naturally or flash dried) (RWC < 10%). The fold increase column shows by what proportion the tensile strength has increased (fold increase above 1.0) or decreased (fold increase below 1.0).

	Tensile strength (MPa)				
Species	**Fully hydrated**	**Naturally dried**	Fold increase from fully hydrated to naturally dried	**Flash dried**	Fold increase from fully hydrated to naturally dried
Arabidopsis thaliana	0.3 ± 0.1 a	0.1 ± 0.02 b	X 0.4	0.2 ± 0.02 b	X 0.6
Zea mays	5.5 ± 0.7 c	7.7 ± 2.1 c	X 1.4	6.1 ± 1.01 c	X 1.1
Craterostigma wilmsii	0.6 ± 0.03 d	1.6 ± 0.2 e	X 2.8	0.1 ± 0.01 f	X 0.2
Sporobolus stapfianus	32.1 ± 1.6 g	36.3 ± 1.7 g (53.6 ± 9.2) **	X 1.1	50.6 ± 5.1 h	X 1.6
Xerophyta humilis	11.8 ± 1.6 i	34.1 ± 3.8 j	X 2.9	28.2 ± 2.1 j	X 2.4
Xerophyta schlecteri	11.1 ± 1.3	> 25.3 *	*	> 25.3 *	*

* Over scale; leaves notched

** Tensile strength of dead *Sporobolus stapfianus* leaves

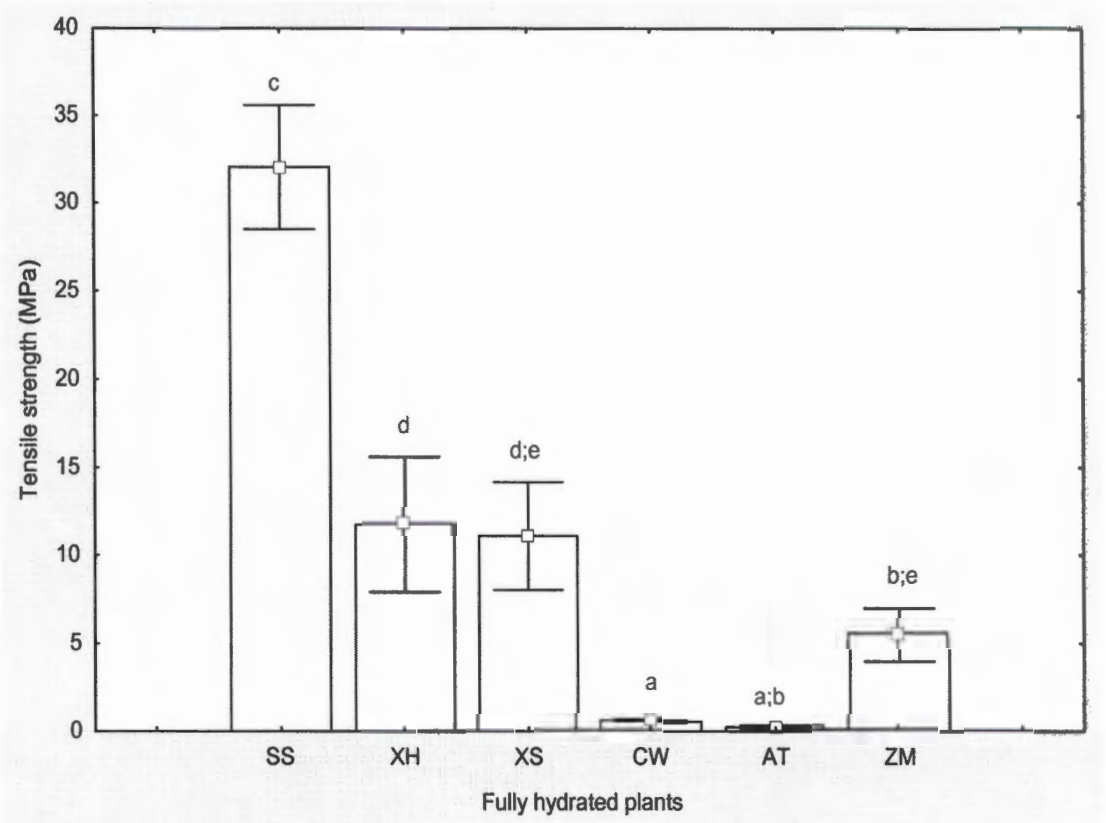
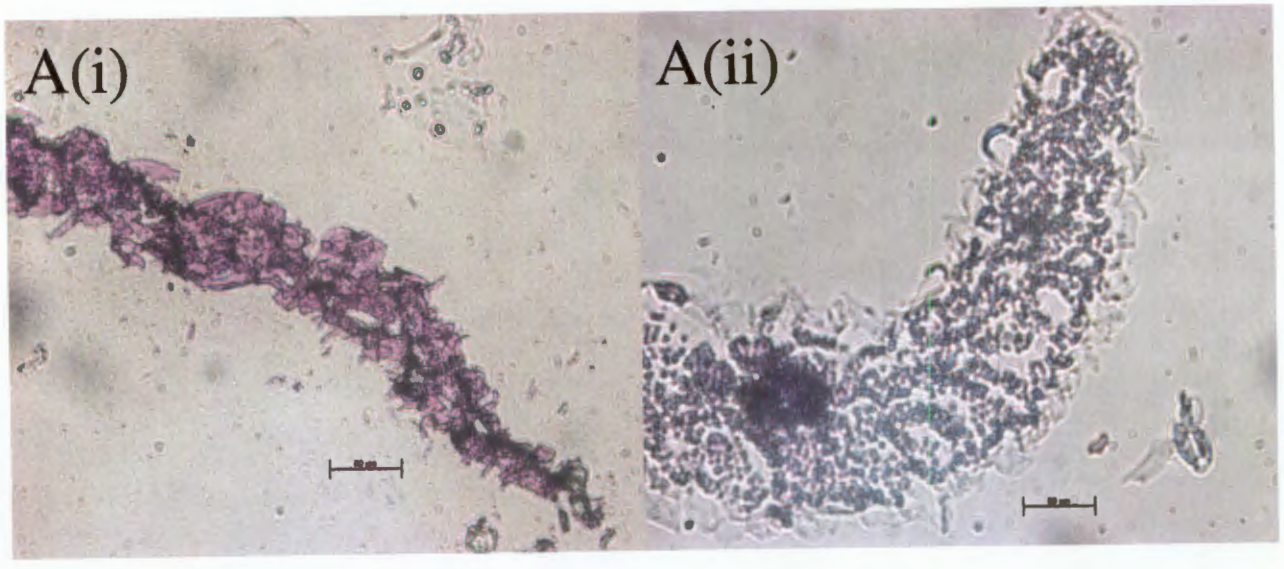
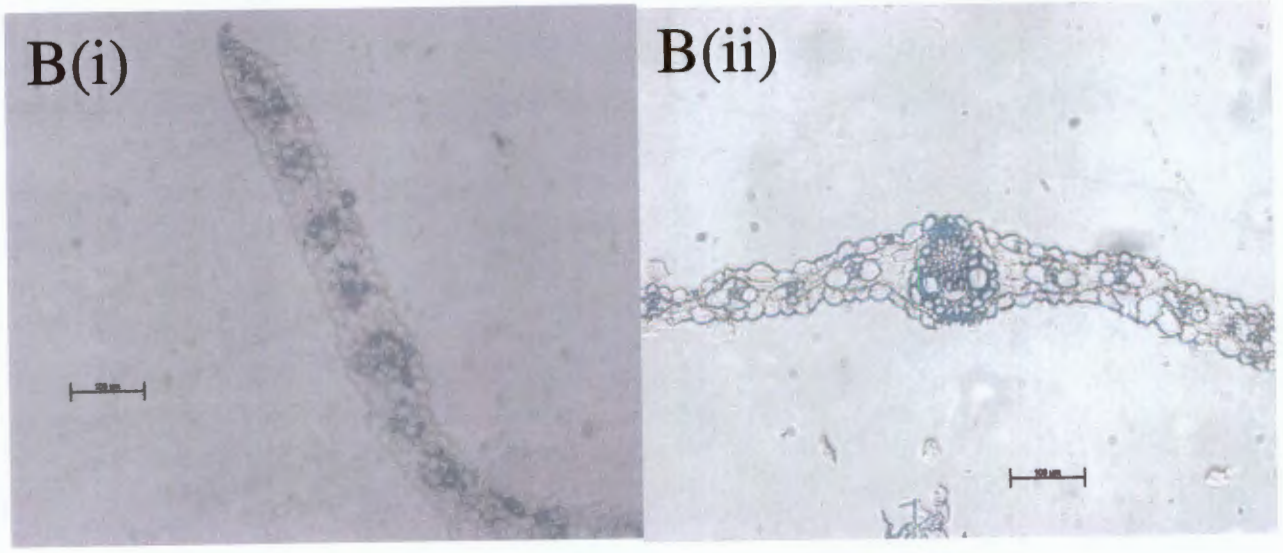
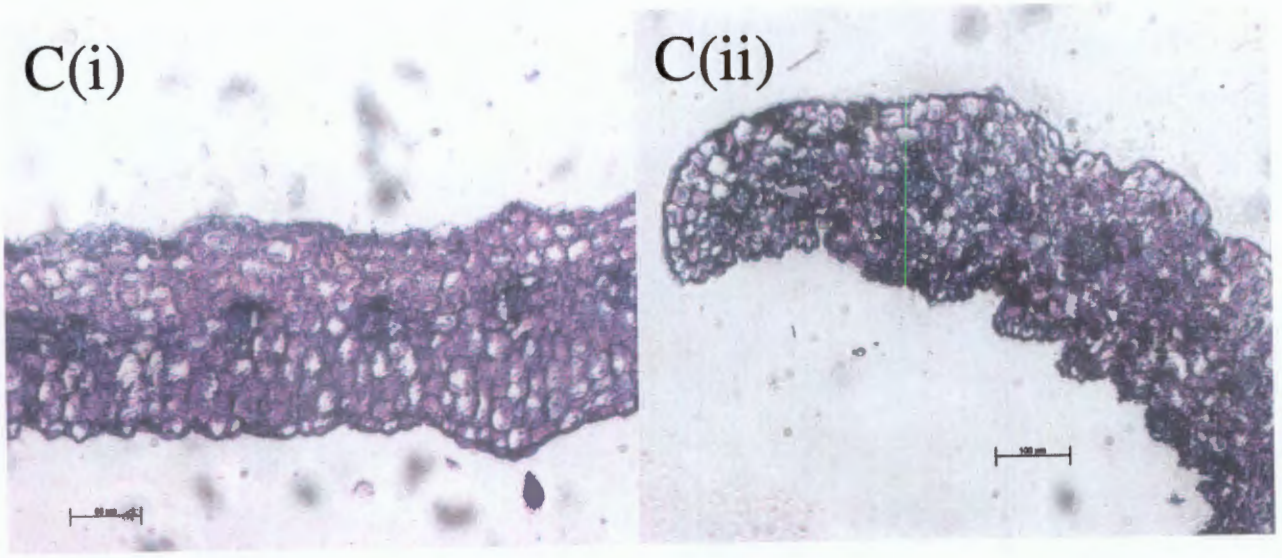


Figure 2: The among species variation of mean tensile strength (± SE) at full hydration. Lower case letters above each bar signify a statistical difference between species ($p < 0.05$), □ = mean values ± SE

(AT = *Arabidopsis thaliana*, CW = *Craterostigma wilmsii*, ZM = *Zea mays*, SS = *Sporobolus stapfianus*, XH = *Xerophyta humilis*, XS = *Xerophyta schlecteri*)


A(i)
A(ii)


B(i)
B(ii)


C(i)
C(ii)

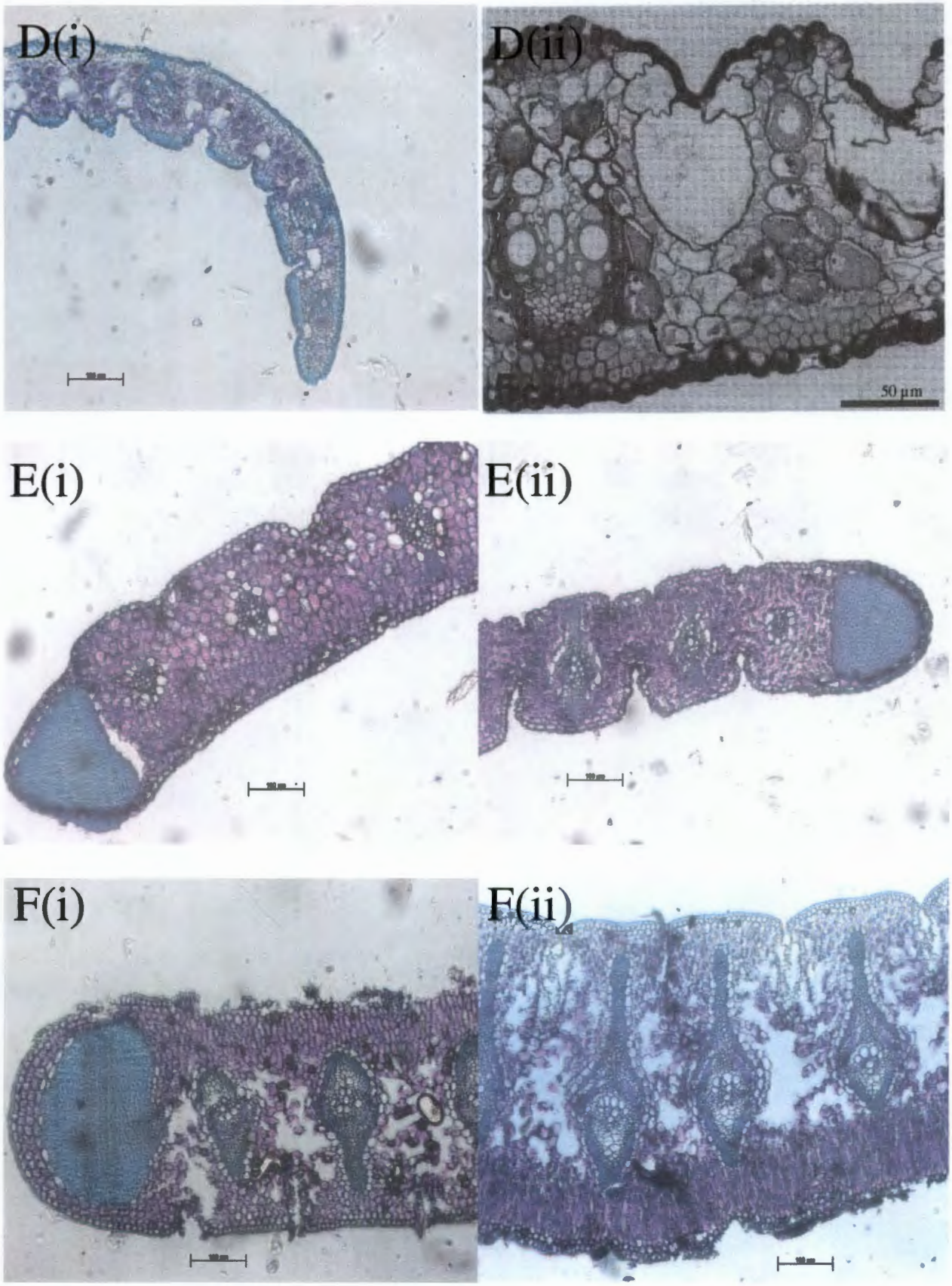


Figure 3: Light micrographs of leaf cross-sections stained with toluidine blue. A = *Arabidopsis thaliana*, B = *Zea mays*, C = *Craterostigma wilmsii*, D = *Sporobolus stapfianus*, E = *Xerophyta humilis*, F = *Xerophyta schlechteri*. Fully hydrated leaves are in the left hand column (i) and naturally dried leaves are in the right hand column (ii). Lignified areas appear light blue while non-lignified areas are purple. Light micrograph D(ii) is taken from Vechhia et al. (1998).

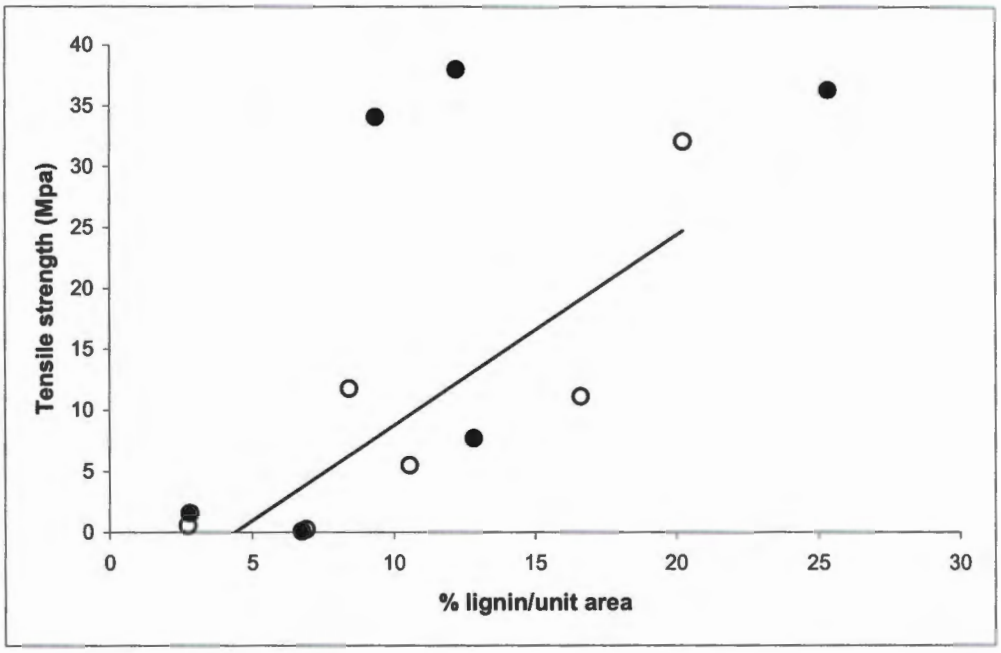


Figure 4: Relationship between % lignin/ unit cross-sectional area and tensile strength for the leaves of fully hydrated plants. Linear regression for fully hydrated leaves (open circles) is y= 1557.72x -6798.68 (R^2 = 0.73). Tensile strength and % lignin values for naturally dried plants (closed circles) show no obvious trend. The combined slope of leaf tensile strength for fully hydrated and naturally dehydrated leaves against % lignin/unit cross-sectional area where y = 1.5241x - 3.7249 and R^2= 0.56. The common slope is significant (F=12.8, df=1;10, p<0.05).

Discussion

Three different mechanisms emerge within the desiccation-tolerant plants from this study. Within the monocotyledonous plants, *X. schlechteri* and *X. humilis* leaf tensile strength increased as RWC decreased whereas the leaf tensile strength of the grass, *S. stapfianus*, did not change when naturally dried, but increased when flash dried (Table 1). Within fully hydrated leaves, a positive correlation existed between the percentage of lignin per unit cross-sectional area of leaf and tensile strength; however, in naturally dried leaves no relationship existed (Figure 4).

Our first hypothesis stated that the tensile strength values of leaves of desiccation-tolerant plants do not change as relative water content (RWC) decreases. The results for *S. stapfianus* agreed with studies by Balsamo *et al.* (2003c) who found that leaf tensile strength of the desiccation-tolerant leaves of the grass, *Eragrostis nindensis*, does not change as RWC decreases, but there is an increase in tensile strength when leaves were flash dried. *E. nindensis* was found to have water, referred to as free-water, possibly held within its vascular bundles when naturally-dried (Balsamo *et al.* 2003c). As *S. stapfianus* is a closely related grass, and the similarity between *S. stapfianus* and *E. nindensis* in terms of tensile strength is apparent, it is possible that *S. stapfianus* has withheld its free-water when naturally dried.

S. stapfianus, as with all grasses, has a unique internal structure with lignified sclerenchyma, the main fibrous tissue, occurring in bundles of fibres and associated with the vascular tissues (Vincent 1982). The vascular tissue accounts for 90-95% of the longitudinal stiffness of the grass leaf. This would explain a higher tensile strength in *S. stapfianus* than *X. humilis* and *X. schlechteri*, the other monocotyledonous plants in this study. Kneebone (1960) suggested that lignin content and different structural arrangements explains differences in the internal structure of leaves. *S. stapfianus* has different leaf architecture and a lignin deposition pattern compared with *X. humilis* and *X. schlechteri*, with the lack of a central midrib but extensive lignification of the epidermal cells. Studies on the tensile properties of *Z. mays* (Balsamo & Orkwiszewski 2004) show that a lack of a midrib, in tips of juvenile as well adult *Z. mays* leaves, caused a decrease in the tensile strength of those leaves. The tensile strength values were, however, found to be higher in the tips of adult compared with

juvenile leaves which was suggested to be because of a higher degree of lignified epidermal cell in the adult leaves (Balsamo & Orkwiszewski 2004). The highest overall tensile strength values in the leaves of *S. stapfianus* may then be due to unusually extensive lignification of the epidermal cells as a result of the absence of a midrib (Figure 3). The presence of a midrib, and extensive lignification on the edges of the leaves in *X. humilis* and *X. schlechteri* (discussed below), but the lack of lignified epidermal cells, may therefore decrease the overall tensile properties of the leaves.

The tensile strength of the leaves of *X. humilis* and *S. stapfianus* increased when flash-dried, agreeing with previous studies (Balsamo *et al.* 2003c), while the dicotyledonous desiccation-sensitive plant (*C. wilmsii*) showed a marked decrease in tensile strength when flash-dried (Table 1). Because protective mechanisms in resurrection plants are induced during drying, it has been suggested that the time taken for this induction prevents survival in rapid (flash) drying (Oliver *et al.*1998). A flash-dried leaf does not recover from desiccation and is thought of as a dead leaf. This was confirmed in this additional experiment where a naturally-dried *S. stapfianus* plant was found not to recover from desiccation. The tensile strength of the initial naturally-dried leaves were not significantly different to the flash-dried leaves (Table 1) supporting the suggestion that flash-dried leaves behave as dead leaves in desiccation-tolerant plants.

The statistically significant increase in the flash-dried leaves of *S. stapfianus* (but no significant increase when naturally dried) may be due to the loss of free-water found in the vascular bundles when naturally-dried (Balsamo *et al.* 2003c). Even though there was an increase in tensile strength from the hydrated leaf to both naturally- and flash-dried leaves of *X. humilis* and *X. schlechteri*, there is no significant difference between natural and flash drying. Tensile strength therefore does not appear to be affected by the type of drying in those plants.

The main difference seems to lie in the dicotyledonous plants where *C.wilmsii* showed a three-fold increase in tensile strength when naturally dried but a four-fold decrease when flash dried. *C.wilmsii* is the only desiccation-tolerant plant that is known to recover from both natural drying and flash drying (Farrant *et al.* 1999).

Studies into the effect of natural and flash drying (Cooper & Farrant 2002) have shown that even though *C. wilmsii* recovers from flash drying some damage is caused. When *C. wilmsii* is naturally dried, its cell walls fold inwards to protect against mechanical stress (Vicre *et al.*1999). This appears to occur in rapidly dried leaves as well but to a lesser extent, resulting in some damage to the cell membrane. In addition, the accumulation of sugars and proteins in the leaves of *C. wilmsii* was reduced in the rapidly dried leaves compared to the naturally dried leaves (Cooper & Farrant 2002). A combination of reduced cell wall folding and reduced sugar and protein accumulation during flash drying, even though not affecting the survival ability of the leaves, appears to have reduced the leaf tensile strength when flash dried.

Figure 2 compares the results from the experiment with those of the controls. *S. stapfianus, X. humilis* and *X. schlechteri* (the monocotyledonous plants) have a higher tensile strength than the monocotyledonous desiccation-sensitive control, *Z. mays*. Correspondingly, *C.* wilmsii has a higher tensile strength than the dicotyledonous desiccation-sensitive control *A.*thaliana. There appears to be a clear difference in tensile strength values between monocotyledonous plants and dicotyledonous plants. The tensile strength of desiccation-sensitive monocotyledonous plants is lower than desiccation-tolerant ones, as is in dicotyledonous plants. The results from this study agree with those from previous studies (Balsamo *et al.* 2003c) that reported lower tensile strength values for the desiccation-sensitive leaves of *E. capensis* and the outer desiccation-sensitive leaves of *E. nindensis* compared with the inner desiccation-tolerant leaves of *E.nindensis*. From the results, it appeared that a possible cause for lower tensile strength in desiccation-sensitive plants might be directly related to the degree of lignification in the leaves (Figure 4).

Vincent (1983) assumed that a piece of homogenous material with a notch in one edge will have a concentration of stress at that point (Figure 5) resulting in the material breaking easily with a small force, for example glass, referred to as a notch-sensitive material.

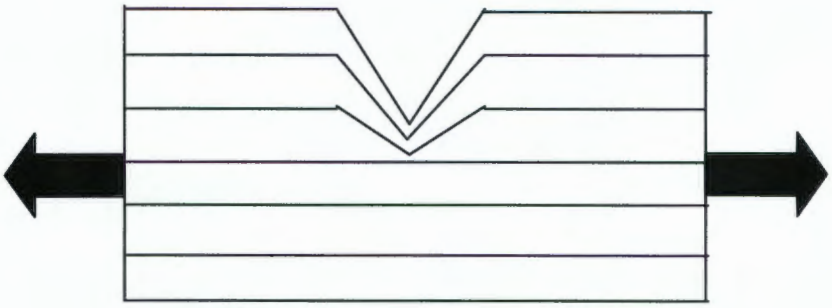


Figure 5: Stress concentration at a notch. In a material under tension (black arrows) a notch will concentrate the stress at the notch (Vincent 1983).

Vincent (1983) attempted to apply a simple mechanical model to grass leaves and in doing so, he showed that grass, with its parallel system of fibres, vascular bundles and cuticle, behaves differently by distributing its stress evenly throughout a leaf. If a notch appears, no stress is concentrated at any one point. This makes grass notch-insensitive even at very low water contents. There is no data for the notch-sensitivity of desiccation-tolerant monocotyledonous leaves.

Figure 6A shows the fracture path of a flash-dried leaf of *X. schlechteri* following notching. It appears that not all monocotyledonous plants behave in the same manner as grass since *X. schlecteri* shows notch-sensitivity even at relatively low tensile forces. The flash-dried leaves of *X. schlechteri* were placed in the clamps of the tensilmeter and at low stress were notched. The leaf fractured immediately on notching. The presence of large areas of lignified tissue in the outer edge of the leaves of *X. humilis* (Figure 3E and 7A) and *X. schlecteri* (Figure 3F and 7B) may explain this. Grasses (*S. stapfianus* in Figure 3), and some other monocotyledonous plants (*Z. mays* in Figure 3) do not have large areas of lignin on the outer edge of their leaves, which probably results in notch-insensitivity. The presence of large amounts of lignin, which has been shown to correlate positively with tensile strength, on the outer edges of *X. humilis* and *X schlecteri*, and relatively little lignin throughout the inner leaf will no doubt affect the load-bearing capabilities of the leaf. A notch through this outer edge appears to generate a fracture. An additional example of the uneven distribution of stress in leaves with large lignin deposits on the outer edge can be seen in Figure

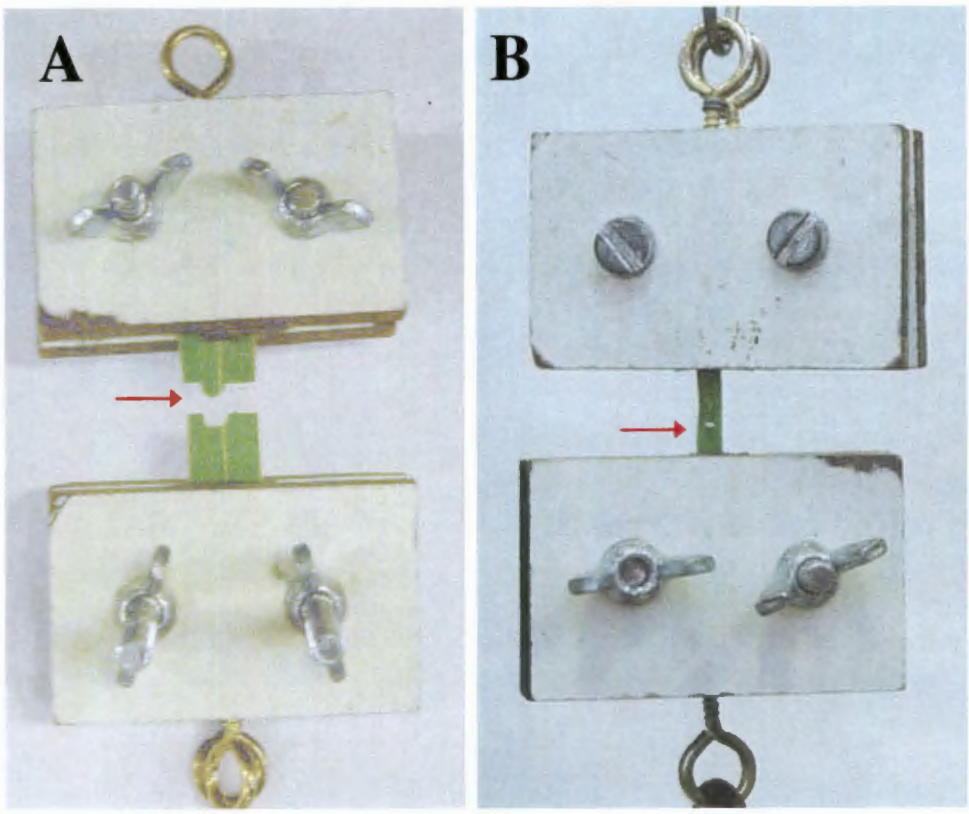


Figure 6: (**A**) A flash-dried *X. sclechteri* leaf clamped in a tensilmeter showing the fracture path following notching (arrowed) (**B**) A fully hydrated *X. humilis* leaf clamped in a tensilmeter showing the initial fracture path (arrowed). The high degree of lignification on the outer edges results in the fracture initialising in the centre of the leaf

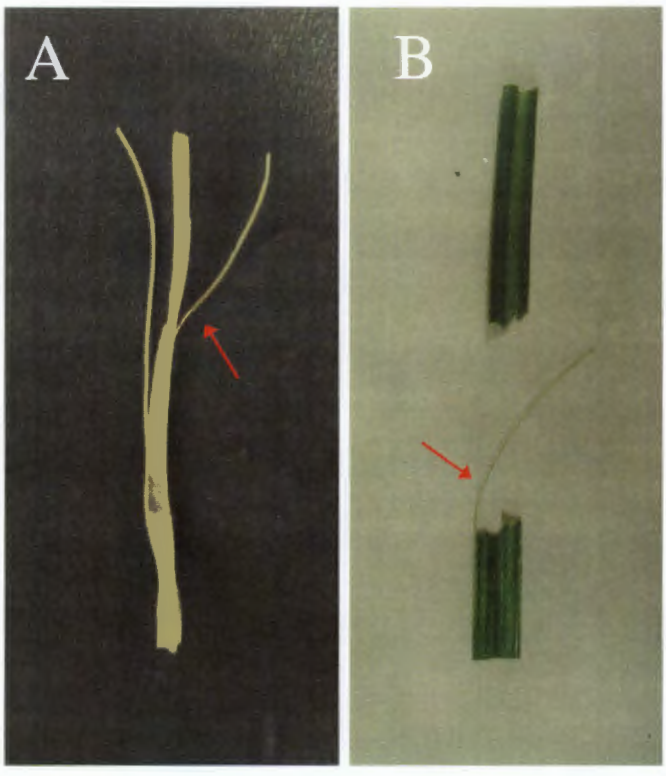


Figure 7: Lamina of (A) naturally dried *X. humilis* and (B) fully hydrated *X. schlechteri* showing thick fibres running along the outer edges (arrowed)

6B where the *X. humilis* leaf, without a notch, fractures in the middle of the leaf (less lignin than the outer edges) which then spreads outwards resulting in complete load-failure.

The second hypothesis of this study stated that tensile strength in leaves is positively correlated with the percentage of lignin per unit area of leaf. Figure 4 shows a clear linear relationship in fully hydrated leaves where an increase in the amount of lignin correlates with an increased tensile strength. The degree of lignification has been shown to decrease from base to tip in the drought-tolerant grass *Eragrostis curvula* (Balsamo *et al.* 2003a) and in *Z. mays* (Balsamo & Orkwiszewski 2004) and the degree of lignification increases with age in *Z. mays* (Balsamo & Orkwiszewski 2004). In both of the above studies an increased degree of lignin corresponded with an increase in tensile strength.

The variation in the amount of lignin deposited in the leaves of the studied species may account for the differences in tensile strength values between species. Large amounts of lignin in a fully hydrated leaf appeared to result in a higher tensile strength (Figure 4). Additionally, a difference in leaf architecture between monocotyledonous plants and dicotyledonous plants might explain the higher leaf tensile strength as monocotyledonous plants tended to have a higher degree of lignification (Figure 4), which could be due to the leaf architecture. Vincent (1982) suggested that a grass leaf is system with sclerenchyma fibres, vascular bundles and cuticle running parallel from tip to base. This parallel system results in a mechanically more stable system than the reticulate venation of dicotyledonous leaves (Cutter 1971).

The lack of a relationship between lignin and tensile strength in naturally-dried leaves may be due to the different protective mechanisms used by the species in this study when desiccated. A further study using only resurrection plants that pack their vacuoles with non-aqueous solute, such as species of the genus *Xerophyta* (Sherwin & Farrant 1996; Farrant 2000), or on resurrection plants that do a combination of cell wall folding and vacuole packing, such as *Myrothamnus flabelifolius* (Farrant 2000), the inner leaves of *E. nindensis* (Balsamo *et al.* 2003b) and *Sporobolus stapfianus*

(Vecchia *et al.*1998) may allow for comparisons of lignin and tensile strength for both fully hydrated and naturally dried leaves.

This study illustrates principally the importance of lignin in the tensile properties of leaves. In addition, the morpho-anatomical role, displayed in the differences in tensile strength between monocotyledonous plants and dicotyledonous plants, and in the difference in tensile strength between desiccation-tolerant and –sensitive leaves is evident. Further studies involving measuring the free-water within the desiccated leaf using proton-NMR and using a more detailed assay to quantifying lignin may help to resolve these differences.

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